RESEARCH PROPOSAL

Quorum Quenching Activity of Endophytes Associated with *Tecomella undulata*

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Introduction

1.1 Endophytes

An Endophyte is an endosymbiont, mostly a bacterium or fungus, which lives inside a plant for at least part of its life cycle (Tan and Zou, 2001). Endophytes are ubiquitous in nature and have been found in all plants studied till now (Gouda *et al*., 2016). Different types of microbial species can be associated with a single plant. Endophytes enter the host plant mainly through the root zone; however, other parts of plants may also be used for entry, such as flowers, stems, and cotyledons. Endophytes within the plant either becomes bounded at the point of entry or spread throughout the host plant, the intercellular spaces or the vascular system. Endophytic population is present more in roots as compared to other parts of plant. Many endophytic species of Bacteria has been isolated from roots, stems, flowers, seeds and fruits of different species of plant (Anjum and Chandra, 2015).

Some endophytes stimulate plant growth by increasing nutrient uptake and nitrogen fixation. They also enhance the plant ability to tolerate abiotic stresses such as drought. They can also be used as an alternative biocontrol agent for controlling bacterial and fungal plant diseases. Endophytes also play an important role in bioremediation, as they have a powerful ability to breakdown complex compounds and thus removing pollutants and wastes from the environment (Nair and Padmavathy, 2014). All endophytes have the ability to decompose complex organic compounds like lignin, cellulose, and hemicellulose and thus they play an important role in nutrient cycling in environment. Endophytic bacteria & fungi are reported to produce many hydrolytic enzymes like cellulases, glucanases, proteases, xylanases, chitinases, amylase, cellulase, L-asparaginase, lipases and caseinases (Urairuj *et al.,* 2003). These enzymes from endophytes are being used for many industrial applications. For example, amylase & cellulase are widely used in food, textile, paper industry and also in bioethanol production.

Endophytes are recognized for the production of different types of secondary metabolic products such as antibacterial, antitumor, antifungal, antiviral, antioxidant and anti-inflammatory compounds (Bell *et al.*, 1995). Many of these bioactive products help to control infection in plants and animals. Endophytes also regulate Quorum Sensing (QS) phenomenon of other prokaryotes. Microorganisms use signaling compounds such as N-acyl homoserine-lactones (AHL) and cyclic peptides for communication and interactions with plants and

animals. Inhibition of QS regulated processes in pathogenic microbes results in reduction in there virulence and this feature can be thus exploited for benefit of environment, plants and humans (Kusari *et al*., 2015). This bioactive nature of endophytes is continuously being explored for human welfare in health sectors for therapeutic purposes.

1.2 Quorum sensing in Prokaryotes

Quorum sensing is a phenomenon by which microorganisms communicate with each other with the help of chemical signaling (Fuqua *et al*., 1994). Quorum sensing enables bacteria to co-ordinate their behavior It is defined as gene regulating phenomenon that occurs in response to cell density and in turn influences many physiological processes of prokaryotes that includes acid tolerance, bioluminescence, biofilm production, pigment production, antibiotic production, sporulation, conjugation, swarming motility, and virulence factor expression (Summerfield, 2012). Quorum sensing system in prokaryotes has been divided broadly into at three different classes (Basavaraju *et al.*, 2016):

- **LuxI/LuxR-type quorum sensing system** which is used by many Gram-negative bacteria that produces N-acyl homoserine lactones (AHL) from *S*-adenosylmethionine (SAM) as signaling molecule. AHLs bind directly to transcription factors and regulate gene expression.
- **Oligopeptide-two-component-type quorum sensing** which is used mostly by Gram-positive bacteria. These bacteria use small molecules of autoinducing peptide (AIP) as their autoinducers. When high level of AIP is detected in the environment, it binds to a receptor to activate kinase. The activated kinase phosphorylates a transcription factor, which results in regulation of gene transcription. Another mechanism involves the transportation of AIP into the cytosol, which binds directly to a transcription factor to initiate or inhibit transcription (Rutherford and Bassler, 2012).
- **LuxS-encoded autoinducer (AI)-2 quorums sensing** is found in both Gram-positive and bacteria. These bacteria synthesize furanosyl borate diester from SAM. Very few biomolecules incorporate boron and AI-2 is one of them. The signaling process relied on several phosphorylation-relay steps. AI-2 is actively transported by the Lsr ABC-type transporter into bacterial cell for its phosphorylation. Transcription of the *lsr* genes is initiated on binding of phosphorylated AI-2 to the transcriptional repressor protein LsrR which is released from the promoter/operator region of the lsr operon (Uroz *et al*., 2008).

1.3 Quorum Quenching

The phenomenon of inhibiting quorum sensing is known as quorum quenching (QQ). It is controlled by different quorum quenching compounds (Dong *et al.,* 2001). These QQ compounds are non- inhibitory to bacterial growth and directly interact with Quorum sensing components. They work enzymatically by degrading signaling molecules or can block the signal generation and signal reception. Most of these quorums quenching compound inhibit the LuxI/LuxR and/or AI-2 quorum sensing systems (Huang *et al*., 2003).

1.3.1. Quorum Quenching Mechanism

On the basis of structure and functions quorum sensing inhibitors can be classified into two groups. First group contains a molecule that looks structurally similar to QS signals like halogenated furanones and synthetic autoinducing peptides which are similar to AHL and AIP signals. The second group contains small chemicals that act as enzyme inhibitors. The Quorum Quenching mechanism in most of the organisms can be divided into different categories as described below:

- **Degradation and inactivation of QS signals**

  The AHL-degrading enzymes are categorized into three categories: AHL-lactonases, AHL-acylases, oxidases/oxidoreductases. AHL-lactonases degrade the AHL- Lactone compounds by hydrolysing one of the bonds of the lactone ring; open ring thus formed inactivates the AHL signals. It is encoded by *aiiA* gene in many bacterial species (Dong *et al*., 2000). AHL-acylases hydrolyses the amide bond of AHLs and thereby produces the corresponding fatty acids and homoserine lactone. It is encoded by *aiiD* gene in many bacterial species (Leadbetter and Greenberg, 2000). Oxidases target the acyl chain by the help of oxidation and reduction process. Therefore, catalyzes a modification of the chemical structure of the signal but not degradation. It is usually found in very few bacterial species (Chowdhary *et al*., 2007).

- **Inhibition of QS signal biosynthesis**

  The produced QS signals can be suppressed by inhibiting the SAM biosynthesis, inactivating synthase enzyme or by blocking the acyl-ACP generation (LaSarre and Federle, 2013).

- **Inhibition of QS signal detection**

  QS Signal detection can be blocked by altering the downstream signal transduction by non-productive signal-receptor complexes which block the binding of native signal in a competitive manner. Moreover, structures of known inhibitors can be modified using computer-aided technologies so that their inhibitory activities are improved or their target specificities are altered (LaSarre and Federle, 2013).

- **Antibiotics as QS inhibitors**

  Studies suggest that QS can also be inhibited by antibiotics in some species. The antimicrobials from plants revealed to influence different behaviors like biofilm formation, bioluminescence production, virulence expression and many more without causing any harm to bacterial cells (LaSarre and Federle, 2013).

1.4 *Tecomella undulata*

Tecomella undulata is commonly found in parts of Rajasthan, Maharashtra, Gujarat, Punjab and Haryana, locally known as **rohida.** It occurs in regions of Thar Desert and therefore also called as **Desert teak**. *Tecomella undulata* belongs to the family *Bignoniaceae*. Due to its stiff, rigid wood it is famous for producing a good quality of timber, which is used in making furniture, wooden crafts, frames, etc. It can bear a large range of temperature ranging from extreme low temperature in winter and high temperature in summers. It has also been used for phytoremediation (Mathur *et al*., 2010). *Tecomella* contains a large number of bioactive compounds like phytosterols, glycosides, tannins, oleanoic acid, Rutin, quercetin, luteolin-7-glucoside, sitosterol, ursolic acid and phenolic compounds (Azam, 1999). These plants possess numerous medicinal properties such as, antibacterial and anti-inflammatory activity, treatment of syphilis, hepatitis, painful swellings and cancer traditionally (Ravi *et al.*, 2011) (Table1). Besides, these, it also acts as blood purifier. *Tecomella* is a very rich source of nutrients like Zinc, lead, chromium, nitrogen, sodium, copper, nickel, magnesium, cadmium, calcium and iron, hence used to treat many deficiency diseases. Highest amount Calcium & iron and negligible amount of cadmium are present in *Tecomella* (Saraf and Sankhla, 2013).

Table1: **Medicinal properties of *Tecomella undulata***

Plant parts	Medicinal property
Bark	Treatment of syphilis, piles, ascites, hepatitis, hepatosplenomegaly, piles, anorexia, flatulence, tumors, worm infestations, eczema, constipation, haematoma, cancer, obesity, jaundice and possess mild relaxant and choleretic activities.
Leaves and stems	Strong HIV prohibitory compounds as ursolic acid, betulinic acid and Oleanolic acid. Treatment of migraine and typhoid fever
Flower	Use for enhancing fertility in women, reduce thirst
Seed	Use to cure hemorrhoids and abscess

Source: (Jain *et al*., 2012; Dhir and Shekhawat, 2012; Ravi *et al*., 2011)

Looking at the medicinal properties of plant parts, it is expected that the endophytes might also be involved in the production of some of the bioactive metabolites which can act as QSIs. Thus, the present study is focused on isolation and characterization of endophytic microbes from *Tecomella undulata* for study of QQ activity.

Review of literature

2.1 Production of Quorum quenching compounds by microorganisms

Quorum sensing has an important role in the biofilm formation and thus contributes to virulence of pathogens. This virulence can be reduced by using compounds that inhibit QS signals. Many such QQ compounds/enzymes have been reported in literature. These quorum quenching compounds plays an important role in controlling infections in animals and plants. They can degrade the quorum sensing signals which are responsible for pathogenicity of microorganisms. Many QS inhibitor bacteria have been reported from different environments like soil, water, marine ecosystem and plant associated rhizosphere and phyllosphere. These environments provide ample nutrients for bacterial growth thus these resident microbes are now greatly explored for studying QS interactions. Three QQ bacteria *Burkholderia* (GG4), *Acinetobacter* (GG2) and *Klebsiella* (Se14) were isolated from the rhizospheric region of *Zingiber officinale* (ginger) collected from the Malaysian rainforest. The GG2 and Se14 strains were reported with broad spectrum AHL-Lactone degrading activity whereas GG4 strain reduced 3-oxo-AHLs into3-hydroxy compounds. It was also reported that the three QQ bacteria attenuated the production of virulence in both human (*P. aeruginosa*) and plant (*Er. carotovora*) pathogens (Chan *et al.,* 2011). In another study, bacteria were isolated from the rhizospheric and phyllospheric region of saffron, fig, and pomegranate plants collected from Iran and screened for QQ activity using *C. violaceum* (CV026) and 3-oxo-C6-HSL. It was reported that maximum bacteria that showed QQ activity belonged to *Pseudomonas* genus. Enzymatic QQ activity was found to be present in both extracellular and intracellular extracts as well as many of them also had non-enzymatic QQ activity. The best biocontrol agents were reported as those with extracellular enzymatic QQ activity (Alymanesh *et al*., 2016). Total 56 marine actinomycetes were isolated from the seawater, collected from Lianyungang region of China. These were screened for the presence of AHL degrading activity, using *C. violaceum* 12472 on LB plates. Five isolates showed AHL inactivation and one strain identified as *Streptomyces parvulus* HY026 was found to be most potent. It was also reported that HY026 strain inhibited the biofilm formation of four bacterial strains namely *P. aeruginosa* PAO1, *Staphylococcus aureus* 95005, *Micrococcus luteus* 95006 and *Ruegeria sp.* 01008 (Miao *et al*., 2017).

Just similar to humans and animals, plants are also continuously exposed to infections by pathogenic microbes. To overcome these pathogens, plants synthesized QS inhibitors (QSI). QQ activity of many plant extracts have already been reported in literature. In a study, 10 traditional Chinese medicine (TCM) herbs were collected from Chinese medical halls, located in Singapore. The extracts of these plants were screened for the QQ activity, using *C. violaceum* CV026 and *P. aeruginosa* PA01 biosensor strain on LB agar plates. It was reported that 7 plant parts exhibited QQ activity. It was also identified that four plants inhibited the swarming motility in PA01 (Koh and Tham, 2011). The buds of *Syzygium aromaticum* (clove) were collected from the local store, located in Malaysia in 2010. It was reported that the chloroform, hexane and methanol extracts of plant inhibited QS activity in biosensor strain *C. violaceum* CV026. Hexane and methanol inhibited violacein production, Methanol and chloroform reduced production of bioluminescence by *E. coli* strain pSB1075. Further, it was also reported that clove extract inhibited QS activity, swarming and pyocyanin production in *Pseudomonas aeruginosa* PA01 (Krishnan *et al*., 2012).

The surface of plants holds large population of bacteria ranging from one micro environment to other. Several studies revealed the presence of AHL-degrading bacteria, isolated from the surface of plants (epiphytes) that inhibited quorum sensing regulated infections produced by the plant pathogens. In a study, Total 413 epiphytes were isolated from the *Solanum tuberosum* L. (potato) roots collected from Japan. These were screened for the presence of AHL degrading activity, using *C. violaceum* CV026 and VIR07 biosensor strain on tryptic soy broth (TSB) medium. It was reported that 61 isolates possessed AHL degradation activity, and most of them belonged to genus *Chryseobacterium* (Rashid *et al*., 2011). Total 1177 epiphytes were isolated from the tobacco leaves and screened for the presence of AHL degrading activity, using *C. violaceum* CV026 on Luria-Bertani (LB) agar. It was reported that 168 isolates possessed AHL degradation activity; these were identified to be species of *Bacillus, Lysinibacillus*, *Acinetobacter*, *Pseudomonas*, *Serratia* and *Myroides* (Ma *et al*., 2013). Total fifty-nine epiphytes were isolated from the Wild berries collected from Ajloun wild forests in 2010. These were screened for the presence of AHL degrading activity, using *C. violaceum* (ATCC 12472). It was reported that six of these isolates possessed AHL degradation activity only, 11 isolates possessed both QSI and antibacterial activity and 42

isolates possessed antibacterial activity. The 3 potent epiphytic strains S130, S153, and S664 were found to be Gram positive spore formers (Abudoleh and Mahasneh, 2017).

QQ phenomenon is not only present in epiphytes but also reported in endophytic microbial population associated with plants. It is suggested that endophytes producing QQIs naturally protect plants from pathogenic bacteria & fungi. Soil samples and plant parts were collected from various regions of Thailand to isolate rhizospheric and endophytic actinomycetes respectively. Total 344 rhizospheric and 132 endophytic isolates were recovered. QQ activity was tested using *A. tumefaciens* NTL4 (pZLR4) (for C8 to C12HSL) and *C. violaceum* CV026 (for C4, C6HSL and 3-oxo-C6HSL) as biosensor strains. About 36.9% of rhizospheric and 51.5% of endophytic actinomycetes showed QQ activity. The maximum HSL-degrading activity was found in endophytic actinomycetes isolate, LPC029 recovered from *Gmelina arborea* Roxb plant. Based on the results of *16S rRNA* gene sequence, the isolate was identified as *Streptomyces*. HPLC analysis revealed its AHL-acylase activity. It also possessed broad substrate specificity ranging from C6- to C12-HSL of which C10HSL was the best substrate. *In vitro* pathogenicity assay conducted with partially purified HSL-acylase showed that it effectively inhibited soft rot disease of potato caused by *Pectobacterium carotovorum* ssp. *Carotovorum* (Chankhamhaengdecha *et al*., 2013).

Total thirty-nine endophytic bacteria were isolated from surface sterilized leaves and bark of *Pterocarpus santalinus* plant collected from forest area of Western Ghats in Karnataka, India. These were screened for the presence of AHLs degrading bacteria, using two biosensor strains (*C. violaceum* CV026 and *E. coli* MG4) by agar overlay assay. Endophytic bacteria *Enterobacter asburiae* PT39 and *B. firmus* PT18 showed potent ability to degrade AHL in their cell free lysates which was evident by inhibition of about 80% violacein production in biosensor strains. Their lysate also inhibited the biofilm formation in *P. aeruginosa* PAO1 and PAO1-JP2. Gene *aiiA* was isolated and amplified which confirmed the presence of AHL lactonase activity. Sequence alignment studies showed that *aiiA* has a "HXHXDH" zinc-binding motif which is being conserved in many other metallohydrolases (Rajesh and Rai, 2013).

The *Silybum marianum* (milk thistle plant) was obtained from Horizon Herbs, located in USA in 2011. The surface sterilized leaves and stem of the plant was grown on malt extract agar. An endophytic fungi (G85) isolated from stem was identified as *Penicillium restrictum*. On

solid medium, the fungus formed distinct red guttates, identified as members of both known and new polyhydroxyanthraquinones. These compounds inhibited QS signals in a clinical isolate of methicillin-resistant *Staphylococcus aureus* (MRSA), with IC50 values in the range of 8 to 120 μM, which suggested the anti-virulence potential of the compounds (Figueroa *et al*., 2014).

Cannabis sativa L. is a medicinal plant that produces cannabinoids drug (Taura *et al*., 2007). Total thirteen endophytic bacteria were obtained from *Cannabis sativa* L. plant collected from Bedrocan BV Company which is licensed to produce Cannabis for medicinal use in Netherlands. The surface sterilized plant material was used for isolation of endophytes on nutrient agar. The quorum quenching activity of endophytic isolates was detected in cell free lysates. Four of the endophytic bacteria were identified to be *Bacillus* sp. strain B3, *Brevibacillus borstelensis* strain B8, *B. megaterium* strain B4 and *Bacillus* sp. strain B11. They were effective in weakening the AHL quorum signals like C6-HSL, C8-HSL, C10-HSL and 3-oxo-C10-HSL of *C. violaceum* at different concentrations. The spatial distribution of QS compounds in *C. violaceum* was studied by advanced analytical techniques like matrix-assisted laser desorption ionization imaging and high-resolution mass spectrometry (MALDI-imaging-HRMS) and it was reported that this biosensor strain releases C6-HSL on colony boundary which then successively diffuses in to agar. The remaining three AHLs were found accruing in the direct vicinity of colony and did not diffuse freely in agar medium. Endophytic bacteria were found to selectively inhibit and modulate all the four QS signals released by *C. violaceum* (Kusari *et al.,* 2014).

Two Endophytic bacterial strains *P. aeruginosa* (XB7 and XB122) and *Stenotrophomonas maltophili*a (XB102) were reported from rhizosphere of *Solanum melongena* (egg plant). The extracellular QQE from them degraded the QS compound 3OH-PAME of a pathogenic microbe *R. solanacearum* to 3- hydroxy-palmitic acid and methanol, revealing its esterase/lipase activity (Achari and Ramesh, 2015). The effect of pH and temperature on enzyme activity was studied. The optimum temperature for QQ esterases from XB7 and XB122 was 37 °C and 28 °C for XB102. Optimum pH of enzyme produced by XB7 and XB122 was 6.8 whereas for XB102, it was reported to be 8.0. The esterase from XB7 and XB122 was found to be of approximately 24 kDa and from XB102 was observed to be 20.0

kDa. The study also reported that the QQ esterase from all three of them remains active in presence of metal ions (as Cu^{2+} and Ca^{2+}) (Achari and Ramesh, 2017).

QS Inhibitors from different sources have been found to be most effective in preventing QS regulated gene expression. The AHL inactivating gene when cloned and expressed in different pathogenic bacteria brings about inactivation or degradation of AHLs. Many studies have been reported suggesting the use of this technique as one of the disease control strategy for plants and humans. About 400 bacterial cultures isolated from soil and chopped plant samples and 100 laboratory strains were screened for AIs degrading activity using *A. tumefaciens* NT1 as biosensor strain. Twenty-four isolates showed AI inactivation of which *Bacillus* strain 240B1 was found to be most potent. The *aiiA* gene responsible for AI inactivation was cloned and fully sequenced. It was expressed in *E. coli* and *Er. carotovora* which is a plant pathogen isolated from Chinese cabbage leaves which showed soft rot symptoms. The study showed that when *aiiA* gene was expressed in transformed *Er. carotovora* SCG1 it weakened the pathogenicity of potato, carrot, Chinese cabbage, eggplant, celery, cauliflower and tobacco (Dong *et al*., 2000). The *aiiA* gene from *Bacillus* A24 strain, which is a known AHL degrader, was incorporated in to the plasmid pME6863 and then introduced in *P. fluorescens* P3 to form P3/pME6863. It was reported that when P3/pME6863 was expressed in different plants, it acted as a biocontrol agent and reduced potato soft rot caused by *Er. carotovora* and crown gall in tomato caused by *A. tumefaciens* and *Bacillus* A24 (Molina *et al*., 2003). An endophyte *Serratia plymuthica* G3 was isolated from the stems of *Triticum aestivum* L. (wheat) in China. It was observed that *S. plymuthica* G3 possesses SplIR and SpsRI type of quorum sensing systems. Liquid chromatography-tandem mass spectrometry of G3 strain and *E. coli* DH5α expressing splI or spsI from recombinant plasmids revealed the production of different types of AHL signals. The AHL regulated biocontrol traits of *S. plymuthica* G3 were then identified by quorum quenching assay using *C. violaceum* CV026. The *aiiA* gene coding AHL-lactonase enzyme from the *Bacillus* strain A24 was expressed in *E. coli* S17-1/pME6863 and then conjugated with G3 strain to obtain G3/pME6863-*aiiA*. Antifungal activity of G3 and its derivatives was tested against *Cryphonectria parasitica*, which is a pathogen that causes chestnut blight disease. It was reported that the production of AHL signals were degraded in the conjugated strain G3/pME6863-*aiiA* that in turn also reduced the antifungal property. It was also reported that

the G3/pME6863-*aiiA* strain also depleted the biofilm formation by *S. plymuthica* (Liu *et al.,* 2011).

3. Objectives

Endophytes are important sources of bioactive secondary metabolites. It has been reported in the literature that about 51% of novel metabolites are endophytic microbial products which is considerably higher than that by other microbes (Schulz and Boyle 2005). In the past few years, endophytes have been considered as a potential source of QS inhibitors as they exist in specialized niches in which they are constantly interacting with different types of microbes harboring the host plant. These necessary and complex interactions are also regularly improved to protect plant against infections by neighboring microbial population. The QSI are widely used now a day for controlling infections in plants, in human health care as an alternative to existing antibiotics and also to control spoilage in food industry and related packaging fields. The literature survey indicates that not much work is carried out on the endophytic population of *Tecomella undulata*. Therefore, taking into consideration the medicinal value of this plant and importance of endophytes, the main objectives of the present study are as follows:

- Isolation and characterization of endophytes from *Tecomella undulata*
- Screening and detection of quorum quenching activity in isolated endophytes
- Extraction and purification of desired metabolite and determination of its chemical nature

4. Materials &Methodology

4.1 Choice of Plant material: *T. undulata* is a multipurpose tree which is used for its timber, fuel wood, fodder and traditional medicine. The plant part possesses number of bioactive compounds that are medically important and used in healthcare (Table 2).

Table 2: Bioactive metabolites of *T. undulata* and their uses

Plant parts	Bioactive compounds	Uses
Roots	6-O-veratryl catalposide	An iridoid glucoside used for tumor treatment and as antiseptic
	Veratric acid	Important role in producing antibiotics and various dyes
Heartwood	Radermachol	Anti-inflammatory activity
	Lapachol	Anticancer, antibacterial, antifungal, antiviral and antitumor activities
	Dehydro-α-lapachone, α- and β-lapachone	An antivascular agent, it induces vascular pruning and growth delay in orthotopic mammary tumors in mice, Anti neoplastic activity, Active against many cancer cell line
	Stigmasterol and β-sitosterol	Antioxidant, hypoglycemic and thyroid inhibiting properties Anti-inflammatory, antineoplastic, anti-pyretic, and immune modulating activities
Bark	Phytosterols	Lowers blood cholesterol thus, reducing the risk of coronary heart disease
	Glycosides	Analgesic, antipyretic, and anti- inflammatory activities, used for treatment of heart disease and act as a natural sweetener
	Phenolic compounds	Antioxidant, anti-carcinogenic or anti-mutagenic and anti-inflammatory effects

Leaves	Oleanoic acid, Ursolic acid and Betulinic acid	Oleanolic and ursolic acid were found to be effective in protecting against chemically induced liver injury in laboratory animals. anti-inflammatory, anti- hyperlipidemic and anti-cancerous properties Both Betulinic acid and Ursolic acid are potent anti-human immunodeficiency virus (HIV) agents
	Tricantanol	Plant growth regulator
	Crisimaritin	A flavonoid with adenosine antagonistic properties in rats
Flower	Rutin	Antibacterial property

Source: (Jain *et al*., 2012; Dhir and Shekhawat, 2012; Ravi *et al*., 2011)

4.2 Sampling site & sample collection

Healthy, asymptomatic, fresh leaves and stems of *Tecomella undulata* will be collected from University of Rajasthan, Jaipur in sterile plastic bags and transported to laboratory for further processing. The botanical identification of plant will be made at herbarium of botany department of University of Rajasthan, Jaipur.

4.3 Surface sterilization

The plant parts collected will be washed under running tap water to remove the adhering soil particles, dust and the surface epiphytes. The initial step of endophytic isolation is surface sterilization of host plant parts. The surface sterilization will be carried out under aseptic conditions by using combinations of sodium hypochlorite (3-5%) (Gardner *et al*., 1982), Tween 20 (2-3 drops), Ethanol (70 %) (Dong *et al.,* 1994) and mercuric chloride (0.01%) (Maroof *et al*., 2012) at different concentrations and time intervals. To confirm process of sterilization, aliquots of sterile distilled water used in the final rinse will be plated onto nutrient medium and plates will be examined for growth after incubation at 37^0C for 2-5days (Anjum and Chandra, 2015).

4.4 Isolation and Morphological Characterization of endophytic microorganisms: The surface sterilized leaves and stems will be air dried in laminar air flow and dissected into small pieces (1cm). These plant parts will be placed on nutrient agar media supplemented

with nystatin (as antifungal agent) for isolation of endophytic bacteria and plates will be incubated at 37 ^{o}C for 24-72 h. Whereas for isolation of endophytic fungi, Potato Dextrose agar media (supplemented with chloramphenicol) will be used and incubation will be carried out at 28 ^{o}C for 4-5 days. All the plates will be monitored regularly for microbial growth. Morphologically distinct bacterial and fungal colonies will be picked up, purified and maintained on nutrient agar and PDA respectively (Maroof *et al.,* 2012). The isolated bacterial endophytes will be studied for their colony characters as pigmentation, form, margin, elevation and size. Shape, arrangement and Grams nature will be studied by Gram's staining. For biochemical and physiological characterization, different biochemical tests will be performed (Cappuccino and Sherman, 2013) and isolates will be identified up to genus level based on Bergey's manual of determinative bacteriology (Holt *et al.,* 2000).The endophytic fungal isolates will be identified on the basis of morphological characteristic that includes the study of their vegetative and sporulating structures by lactophenol cotton blue mounting (Aggarwal and Hasija, 1980; Domsch *et al.,* 1980).

4.5 Screening of isolates for Quorum quenching activity

4.5.1 Biosensor strains: All biosensor strains will be purchased from Microbial Type Culture Collection and Gene Bank (MTCC) Chandigarh, India.

- ***Chromobacterium violaceum* (ATCC 12472/MTCC 2656)**, will be used as indicator strain to study QQ activity of isolates. *C. violaceum* is a Gram-negative bacterium that produces non-diffusible violacein antibiotic. Its production is controlled by vio ABCD operon (August *et al.,* 2000). It constitutively expresses C10HSL signal molecule which when binds with its specific receptor, induces the production of purple colored violacein pigment giving purple colour to its colony (Morohoshi *et al*., 2008). Compounds with QS inhibitory potential are able to inhibit the production of violacein pigment (Adonizio *et al*., 2006).
- ***C.violaceum* CV017**: This is a mutant derived from *C. violaceum* (ATCC 12472) which forms pigment on exogenous supply of C10-C16-HSL and particularly by C10-HSL.
- ***C.violaceum* CV026**: It is a Mini-Tn5 mutant derived from C. *violaceum* (ATCC 31532) and form pigment only on exogenous supply of AHLs with acyl chains from four to eight carbons (C4-C8-HSL).
- ***Escherichia coli DH5α***: It will be used as a negative control in all studies (Chan *et al*., 2011).

- ***Bacillus cereus*** (MTCC 1272): This will be used as a positive control in the AHL inactivation studies. It degrades AHL by targeting the lactone bond and amide linkage by production of AHL lactonase enzyme which is encoded by *aiiA* gene homologues (Chong *et al*., 2012).
    - All the biosensor strains will be maintained in LB plates at 30 □ supplemented with kanamycin (25 µg/ml).
    - *E. coli DH5α* will be grown at 37 ^{o}C supplemented with ampicillin (100 µg/ml).
    - *B. cereus* will be grown in LB medium at 28 °C.

4.5.2 Detection of QQ activity

a) **Plate screening assay** Plate screening assay will be used to study quorum quenching activity with *C. violaceum* (MTCC 2656) as an indicator. LB agar plates will be spreaded with *C. violaceum* culture and test endophytes will be spot inoculated on them. Plates will be incubated for 48 h at 30 °C, and quorum sensing inhibition will be detected by presence of a ring of colourless, but viable, cells around the bacterial isolate colony (Adonizio *et al*., 2006).

b) Soft agar overlay method

Inhibition of violacein production in mutants of *C. violaceum*CV026 and CV017

In order to determine the spectrum of QQ activity mutants of *C. violaceum* will be used. The isolated endophytic bacteria will be tested for their QQ activity in solid plate assays carried out as per the protocol given by Mc Clean *et al*., 1997 using soft agar overlay plates. *C. violaceum* CV026 will be grown in LB broth medium for 24 h, diluted (1/100) and 5 ml of it will be added to soft LB agar (0.8%). This mixture will be immediately overlaid over LB agar plates (1.5% agar) and wells will be punched into these agar plates.

For QQ assay the endophytic isolates will be grown in LB medium for 24 h under shaking conditions. Stock solution of N hexanoyl-L-homoserine lactone (C6-HSL) will be prepared in water and added to 1 ml of bacterial culture grown previously to achieve concentration of 2µg/ml and incubated further for 24 h. After incubation, the culture medium will be centrifuged at 4□ and supernatants will be obtained. For studying inhibition of C6-HSL activity, 50 µl of supernatants obtained will be loaded in wells punched in to soft agar overlay plates and incubated for 24 h to observe inhibition of pigment production. Positive results will be indicated by appearance of a ring of colorless but viable cells around the test colony.

Sterile solution of C6-HSL and water will be used as controls. All positive isolates will be further tested for their activity to degrade N-decanoyl-Lhomoserine lactone (C10-HSL) using *C. violaceum* VIR07 (Morohoshi *et al*., 2008) by same methodology as described above. Potent isolates will be selected by comparing the zone of colourless ring obtained around wells.

4.6 Extraction of QQ metabolite and Pigment inhibition assay

The potent endophytic isolates showing QQ activity will be grown in broth medium for 48-72 h. After incubation the culture medium will be centrifuged at 10,000 rpm for 10 min at 4□. Supernatants will be extracted using different solvents (Hudson *et al.,* 2010). Solvent will be evaporated and concentrate will be stocked for further testing.

In order to quantify violacein production, pigment inhibition assay will be carried out. The *C. violaceum* (MTCC 2656)will be cultured in LB broth medium for overnight. One milliliter of this bacterial culture will be added to separate flasks containing LB broth medium supplemented with QQ extracts and will be incubated in an orbital shaker at 30□and 150 rpm for 18-20h. Control flask will be maintained simultaneously that has sterile distilled water instead of *C. violaceum.* After incubation, the cultures will be centrifuged 10,000 rpm for 30 min. DMSO will be used to dissolve the violet pigment present in bacterial cells in pellet. The cell debris in resulting solution will again be removed by centrifugation for 10 min at 10,000 rpm. OD of the resulting solution will be measured at 570 nm and inhibition rate will be calculated as compared to control.

4.7 Determination of nature of QQ activity and purification of active metabolite

Protein or non-protein nature of the extract will be determined. The active fraction of the extract will be incubated with Proteinase K (1 mg/ml) for 18 h at 37□. After incubation, the activity of the extracts will be tested using well diffusion method as described above.

Chromatographic techniques will be used for fractionation and purification of extract (if non proteinaceous nature) like TLC, HPLC etc. The chemical nature of extract can then be identified using suitable spectroscopic techniques like FTIR, GC-MS (if non proteinaceous nature) (Bucar *et al*., 2013). If QSI is found to be of proteinaceous nature then it can be partially purified by ammonium sulphate precipitation method and characterized.

4.8 Identification of potent isolate

The isolates showing maximum pigment inhibition activity will be identified by 16S/18S rDNA sequencing.

4.9 Inhibition of QS-Mediated Virulence Determinants

In order to study the application of bioactive QQ extract, biocontrol assays will be carried out to inhibit QS mediated virulence determinants in any one of the plant pathogens (like soft rot causing *Pectobacterium carotovorum* in potato tubers) (Dong *et al*., 2004) or any one human pathogen (like swarming motility inhibition in *Pseudomonas aeruginosa*) (Kuchma *et al.,* 2015). The entire work plan proposal is shown below (Fig. 1).

WORK PLAN

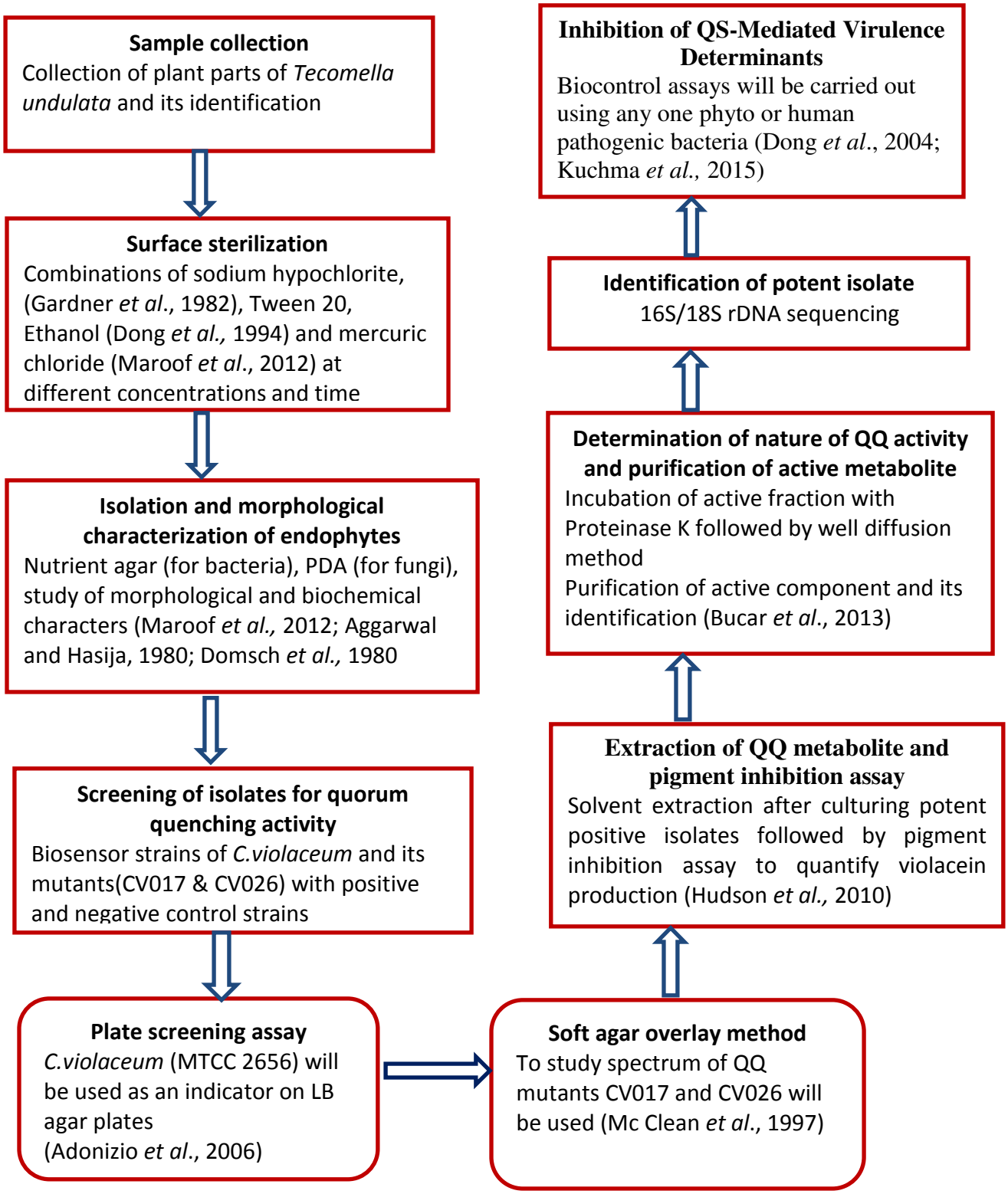
Sample collection
Collection of plant parts of *Tecomella undulata* and its identification
Surface sterilization
Combinations of sodium hypochlorite, (Gardner *et al*., 1982), Tween 20, Ethanol (Dong *et al.,* 1994) and mercuric chloride (Maroof *et al*., 2012) at different concentrations and time
Isolation and morphological characterization of endophytes
Nutrient agar (for bacteria), PDA (for fungi), study of morphological and biochemical characters (Maroof *et al.,* 2012; Aggarwal and Hasija, 1980; Domsch *et al.,* 1980
Screening of isolates for quorum quenching activity
Biosensor strains of *C.violaceum* and its mutants(CV017 & CV026) with positive and negative control strains
Plate screening assay
C.violaceum (MTCC 2656) will be used as an indicator on LB agar plates
(Adonizio *et al*., 2006)
Soft agar overlay method
To study spectrum of QQ mutants CV017 and CV026 will be used (Mc Clean *et al*., 1997)
Extraction of QQ metabolite and pigment inhibition assay
Solvent extraction after culturing potent positive isolates followed by pigment inhibition assay to quantify violacein production (Hudson *et al.,* 2010)
Determination of nature of QQ activity and purification of active metabolite
Incubation of active fraction with Proteinase K followed by well diffusion method
Purification of active component and its identification (Bucar *et al*., 2013)
Identification of potent isolate
16S/18S rDNA sequencing
Inhibition of QS-Mediated Virulence Determinants
Biocontrol assays will be carried out using any one phyto or human pathogenic bacteria (Dong *et al*., 2004; Kuchma *et al*., 2015)

Fig. 1: FLOW CHART SHOWING WORK PLAN

5. Significance of the study and expected outcome

Quorum sensing is used by bacteria in order to regulate different physiological activities like establishment of virulence, cell to cell communication, formation of biofilms and antibiotic resistance. In the present study, an attempt will be made to show if quorum quenching can be used by *T. undulata* associated endophytes as an anti-virulence strategy that can disrupt cell to cell quorum sensing signals in the biosensor strain, *C. violaceum*. This in turn might provide an insight into anti-virulence strategies used by endophytes that help them to survive inside plants. These endophyes can in future be studied to control bacterial pathogens and might open up alternate paths to combat the increasing bacterial resistance to current antibiotic therapy.

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